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13. ABSTRACT *(Maximum 200 Words)*

We are developing a simple and effective method for the delivery of cancer vaccines by noninvasive vaccination onto the skin (NIVS) using a patch. The hypothesis is that a noninvasive vaccine patch can elicit specific immune responses to tumor-associated antigens with resultant eradication of limited numbers of tumor cells in animals with low tumor burden breast cancer. In these studies, we have elicited anti-CEA antibodies by topical application of an adenovirus vector encoding CEA. Furthermore, animals immunized by this novel vaccination modality were well protected against a mammary tumor cell line expressing CEA. When compared to other means of immunization including intramuscular injection of DNA and intranasal inoculation of adenovirus vectors, the skin-targeted vaccine patch appeared to be more protective in a disease setting, probably due to the immunocompetence of the outer layer of skin where antigens were expressed. We envision that patch-based vaccination may emerge as an important technique for the administration of vaccines because the procedure is simple, effective, economical, painless, and safe. It may also boost vaccine coverages due to patient comfort.

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Table of Contents

INTRODUCTION

The induction or augmentation of tumor-specific immune responses providing protection against neoplastic disease is a promising approach for treating metastatic breast cancers. Genetic immunization potentially may present functional antigenic proteins to the host for recognition by all arms of the immune system, yet is able to delete pieces of tumor antigens that may have deleterious effects. We have demonstrated that the surface of the skin is a convenient site for the inoculation of genetic vaccines. Since the outer layer of skin interfaces directly with the external environment and is in constant contact with innumerable pathogens, immunologic components for the elicitation of both humoral and cytotoxic cellular immune responses must be present along the skin border for counteracting undesirable infections. Evidence supporting the immunologic competence of the outer layer of skin includes: 1) Antigens expressed in the epidermis are more immunogenic than those expressed in the dermis (Eisenbraun et al., 1993), and 2) genetic vaccines inoculated into the epidermis using a gene gun are more potent than those injected intramuscularly (Fynan et al., 1993). Injection of vaccines underneath the epidermis as commonly practiced is likely to bypass an epidermis-associated immune surveillance zone along the border, resulting in insufficient or inappropriate immune responses. The large accessible area of the skin and its durability are other advantages for applying genetic vaccines to this tissue. The immunologic competence of the skin, the ease with which genes can be targeted to defined sites within the skin, the rapid turn-over of skin cells, and our finding that animals can be protected against tumor challenges by noninvasive vaccination onto the skin (NIVS), may allow for the development of a unique method for the administration of vaccines. In these studies, we have demonstrated that anti-CEA (carcinoembryonic antigen) antibodies could be elicited by adenovirus-based NIVS. Furthermore, animals immunized by a skin patch containing AdCMV-hcea (an adenovirus vector encoding human CEA) (Tang et al., 1997b) were protected against challenges by a mammary tumor cell line expressing CEA. Results suggested that vaccination against metastatic breast cancers may be achieved by a noninvasive skin patch. This approach not only may boost vaccine coverages because the procedure requires no specially trained personnel and equipment, but also may be able to elicit potent antitumor immune responses because antigens are expressed in the outer layer of skin which is a very immunocompetent tissue.

BODY

***Task 1.* Construction of adenovirus recombinants for gene painting.**
Adenovirus vectors with all viral genes deleted (gutless adenovirus vectors) were developed using the Cre-loxP recombination system (Parks et al., 1996). These vectors not only accommodate large inserts, but also may allow repeated expression of antigens in animals due to their reduced immunogenicity (Chen et al., 1997). A gutless adenovirus vector system (*i.e.*, pRP1001, AdLC8cluc, and 293Cre4 cells) has been made available to us from the Merck Research Laboratories. However, a problem with the current gutless adenovirus system is the contamination of the viral stock by helper virus (Parks et al., 1996) because efficiency of the Cre-loxP recombination system for excising the packaging signal is not 100%. Our vector preparation contained substantial amounts of the AdLC8cluc helper virus during our preliminary studies. Although the vector/helper ratio may be improved after prolonged propagation in 294cre cells due to a selective disadvantage for the helper virus to be packaged, the procedure is time-consuming and the viral stock can hardly be helper-free. To this end, we are taking a novel approach for constructing helper-free gutless adenovirus vectors by co-transfecting pRP1001-based plasmids with a packaging signal (Grable and Hearing, 1992)-free helper plasmid. To construct a helper plasmid without a packaging signal (AdEasy-hp), the left ITR (inverted terminal repeat) of adenovirus was amplified from the pShuttle plasmid (He et al., 1998) using two primers 5'-CGGGGATCGATGGCGCATCATCAATAATATACCTTATTT-3' and 5'-ATATCGATACAACATCCGCCTAAAACCGCGCG-3'. pAdEasy-hp was constructed by cloning the left ITR sequence into the unique Cla I site of the pAdEasy-1 plasmid which lacks the left ITR, packaging signal, and E1 region (He et al., 1998). When pRP1001-based plasmids are co-transfected with pAdEasy-hp into human 293 cells, the E1 function will be provided *in trans* by the host whereas all other adenoviral proteins may be produced by pAdEasy-hp. Because pAdEasy-hp contains two functional ITRs as the origin of replication, it may replicate as an autonomous replicon in 293 cells (Hay et al., 1984), and late gene transcripts may be terminated correctly after DNA synthesis. Since pAdEasy-hp does not contain a packaging signal, none of its DNA molecules can be packaged into infectious particles. pAdEasy-hp may thus support the production of helper-free gutless adenovirus vectors.

***Task 2.* Develop DNA-based gene painting schemes.**
We are the first to have demonstrated that an immune response could be elicited by topical application of adenovirus recombinants (Tang et al., 1997b), DNA:adenovirus complexes (Shi et al., 1999), or DNA:liposome complexes (Shi et al., 1999). Others have shown that the immune system could also be activated to some extent following topical application of cholera toxin protein (Glenn et al., 1998) or naked DNA (Fan et al., 1999). However, emerging evidence suggests that the adenovirus-based vector system is more promising than the DNA-based system as a carrier for skin-targeted noninvasive vaccines because antigen expression from an adenovirus vector is more efficient than DNA-mediated gene expression following topical application (Shi et al., 1999). The problem of eliciting an anti-adenovirus immune response which may interfere with subsequent cycles of immunization following topical application of the

E1/E3-defective adenovirus vector may readily be circumvented by the development of the helper-free gutless adenovirus vector system (Task 1).

***Task 3.* Construction of ubiquitin-based expression vectors for gene painting.**
Since T cells recognize short peptides presented by MHC class I molecules, and since ubiquitin-dependent proteolysis degrades endogenously synthesized antigens and generation of short peptide ligands (Ciechanover, 1994), it is thus logical to use ubiquitin conjugation to target antigens into the ubiquitin-proteasome degradation pathway for MHC class I-restricted antigen processing and presentation. However, recent experimental evidence showed that fusion of antigens to ubiquitin could be counterproductive in eliciting an immune response for reasons not well understood (Fu et al., 1998).

***Task 4.* Validation of new adenovirus recombinants.**
We have shown that a potent immune response could be elicited following topical application of an E1/E3-defective adenovirus vector encoding CEA (Tang et al., 1997b). A novel gutless adenovirus vector encoding CEA shall soon be tested for its efficacy in inducing an anti-breast cancer immune response.

***Task 5.* Construction of target cell lines.**
The murine mammary tumor cell line JC derived from a female Balb/c mouse was obtained from ATCC. A CEA-expressing mammary tumor cell line JC-hcea was constructed by co-transfecting pGT37 (Conry et al., 1994) with pHβAPr-1-neo (Gunning et al., 1987) at a molar ratio of 10:1, followed by selecting transfectants in medium containing G418. G418-resistant clones containing the human CEA sequences were validated by PCR analysis.

***Task 6.* Vaccination of animals against mammary tumors by gene painting.**
1. Elicitation of anti-CEA antibodies in mice. We have experience in immunizing animals with a noninvasive vaccine patch (Shi et al., 1999; Tang et al., 1997b). **Figure 1** shows that antisera against CEA could be induced in mice by a vaccine patch containing AdCMV-hcea (an adenovirus vector encoding CEA) (Tang et al., 1997b). Serum samples were collected from BALB/c mice that had been immunized by intramuscular injection of pGT37 DNA (a plasmid expression vector encoding human CEA) (Conry et al., 1994), intranasal inoculation of AdCMV-hcea, or noninvasive application of AdCMV-hcea onto abdominal skin using a skin patch. Evidence suggested that only a small fraction of topically applied vectors may be absorbed by the skin (Shi et al., 1999). **Figure 1** shows that absorption of vectors by unbroken skin could elicit anti-CEA antibodies at a higher titer than that achieved by intramuscular injection of a large dose of DNA, although intranasal inoculation of adenovirus vectors appeared to be more potent than NIVS in eliciting a humoral immune response probably due to more efficient gene transfer in the respiratory tract. Control animals including naïve mice and mice immunized by topical application of an irrelevant vector all failed to elicit anti-CEA.
2. Elicitation of a protective antitumor immune response by skin-targeted noninvasive vaccine patches. To test the efficacy of a noninvasive vaccine in a

disease setting, mice were challenged by subcutaneous injection of 3 X 10^5 JC-hcea cells, then monitored daily for mortality. **Figure 2** depicts mice immunized by AdCMV-hcea-based noninvasive vaccine patches compared to groups which were immunized by intramuscular injection of pGT37 DNA, intranasal inoculation of AdCMV-hcea, topical application of an irrelevant vector AdCMV-PR8.ha, or received no vaccines. Mice immunized by topical application of AdCMV-hcea were afforded 100% protection from the challenge. Animals immunized by intranasal inoculation of AdCMV-hcea or intramuscular injection of pGT37 were also protected. In contrast, those that were immunized by an irrelevant vector or received no vaccines had the highest mortality rate and sustained significant weight loss before they either died, or slowly recovered. It is interesting to note that protection did not correlate with the titer of anti-CEA (**Figure 1**). It is conceivable that a protective antitumor immune response may also involve cytotoxic T lymphocyte (CTL) responses that have not been measured in these studies.

3. In vivo cytotoxicity assay. To analyze the antitumor immune response in an in vivo setting at an early stage following tumor challenge, JC-hcea cells were grown on a small disk and implanted onto muscle as described (Tang et al., 1996). **Figures 3A** and **3C** show histologically that JC-hcea cells proliferated from a monolayer to a tumor nodule after 5 days of in vivo growth in a naïve animal. In contrast to the naïve control, the implanted JC-hcea cells were nearly eradicated after 5 days of in vivo growth in animals immunized by topical application of AdCMV-hcea (**Figures 3B** and **3D**). Moreover, a large number of immune effectors infiltrated into the implantation bed concomitantly with the eradication of breast tumor cells (**Figures 3B** and **3D**). Histologic evidence thus supports the hypothesis that the death of tumor cells was mediated by a potent antitumor cellular immune response.

4. Relocation and degradation of foreign DNA after localized gene delivery in a noninvasive mode. In an attempt to determine whether topical application of an adenovirus vector could deliver foreign DNA beyond the inoculation area, we extracted DNA from various tissues, followed by amplification of the transgene as well as the adenovirus type 5 fiber gene by PCR after noninvasive delivery of AdCMV-luc (Tang et al., 1997a) into neck skin. As shown in **Figure 4**, the full-length luciferase and fiber genes could be amplified from neck skin 3 hours post-inoculation. The full-length gene was usually undetectable in neck skin DNA after 1 day or in DNA extracted from other tissues. However, subfragments of both luciferase and fiber genes could be amplified from liver, whole blood, ear, abdominal skin, or lymph nodes using different sets of primers. No foreign DNA was detectable in any of the tissues 4 weeks post-inoculation. Results suggested that topical application of an adenovirus vector could deliver foreign DNA into a localized area in skin, although foreign DNA may be rapidly acquired by other cell types, degraded, and relocated into deep tissues. The elimination of foreign DNA in 4 weeks highlighted the safety of NIVS.

***Task 7.* Compare gene painting with other modes of genetic immunization.**

We have compared gene painting with intramuscular injection of DNA-based vaccines. As shown in **Figures 1** and **2**, topical application of 10^8 pfu AdCMV-hcea was more potent in eliciting an anti-CEA antibody response as well as a protective immune response against tumor challenge than intramuscular injection of 100 μg (equivalent to 10^{13} copies) pGT-37 DNA. We envision that only a small number of AdCMV-hcea

particles could be absorbed by skin following topical application of 10^8 pfu. Results provide solid evidence that the outer layer of skin is more immunocompetent than muscle, and the surface of skin is an effective target site for vaccine administration.

KEY RESEARCH ACCOMPLISHMENTS

- Topical application of an adenovirus vector is capable of protecting animals against a lethal dose of tumor challenge. Although several different laboratories have shown that a humoral immune response could be elicited by topical application of adenovirus vectors (Tang et al., 1997b), DNA:adenovirus complexes (Shi et al., 1999), DNA:liposome complexes (Shi et al., 1999), naked DNA (Fan et al., 1999), or cholera toxin protein (Glenn et al., 1998), this is the first demonstration that topical application of vaccines without causing tissue damage is able to protect animals against cancers in a disease setting.
- The in vivo cytotoxicity assay (Tang et al., 1996) provided evidence that the eradication of breast tumor cells in vivo may have been mediated by a potent antitumor cellular immune response.
- We have demonstrated that the outer layer of skin is more immunocompetent than muscle. This observation makes biological sense because the outer layer of skin is in frequent contact with environmental pathogens, and should be the focus of immunosurveillance.
- Construction of a helper-free gutless adenovirus system is underway.
- We have demonstrated that the skin does not allow environmental DNA to persist. Subfragments of degraded vector DNA could traffic to a variety of tissues following topical application, presumably via antigen-presenting cells.

REPORTABLE OUTCOMES

- **Publication:** Shi, Z., Curiel, D. T., and Tang, D. C. (1999). DNA-based non-invasive vaccination onto the skin, Vaccine *17*, 2136-2141.
- **Oral Presentation:** Skin-targeted noninvasive vaccination against mammary tumor cells. *Era of Hope Department of Defense Breast Cancer Research Program Meeting.* Atlanta, Georgia, June 11, 2000
- **Oral Presentation:** Analysis of target cells following vector-based noninvasive vaccination onto the skin. *The Third Annual Meeting of The American Society of Gene Therapy.* Denver, Colorado, June 3, 2000
- **Oral Presentation:** Skin-targeted noninvasive vaccination. *National Vaccine Advisory Committee.* Washington, D.C., May 22, 2000
- **Oral Presentation:** Skin-targeted non-invasive vaccine patches. *2000 Annual Meeting of the Society for Investigative Dermatology.* Chicago, Illinois, May 13, 2000
- **Oral Presentation:** Skin-targeted noninvasive influenza vaccines. *Second Annual Meeting of the American Society of Gene Therapy.* Washington, DC, June 10, 1999
- **Patent:** Allowance of the U.S. patent "Vaccination by topical application of genetic vectors" (U.S. Serial No. 09/402,527)
- **Special Award:** Year 2000 Wallace H. Coulter Award for Innovation and Entrepreneurship with a stipend of $100,000
- **Preceptor for Dr. Mingtao Zeng's Postdoctoral Fellowship:** "Development of a skin-targeted vaccine patch against anthrax" (Dermatology Foundation, 07/01/00-06/30/01, $25,000)

CONCLUSIONS

We have demonstrated that noninvasive application of an adenovirus vector encoding a tumor-associated antigen onto the skin could elicit an immune response against the antigen, and protect vaccinees against tumor challenges. Evidence suggested that the efficacy of a "vaccine patch" may be even greater than that achievable by the commonly used intramuscular route, possibly due to the immunocompetence of the outer layer of skin. We envision that a noninvasive vaccine patch may emerge as a novel vaccination modality in a few years because the procedure is simple, effective, economical, painless, and safe. It may also boost vaccine coverages due to patient comfort.

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APPENDICES

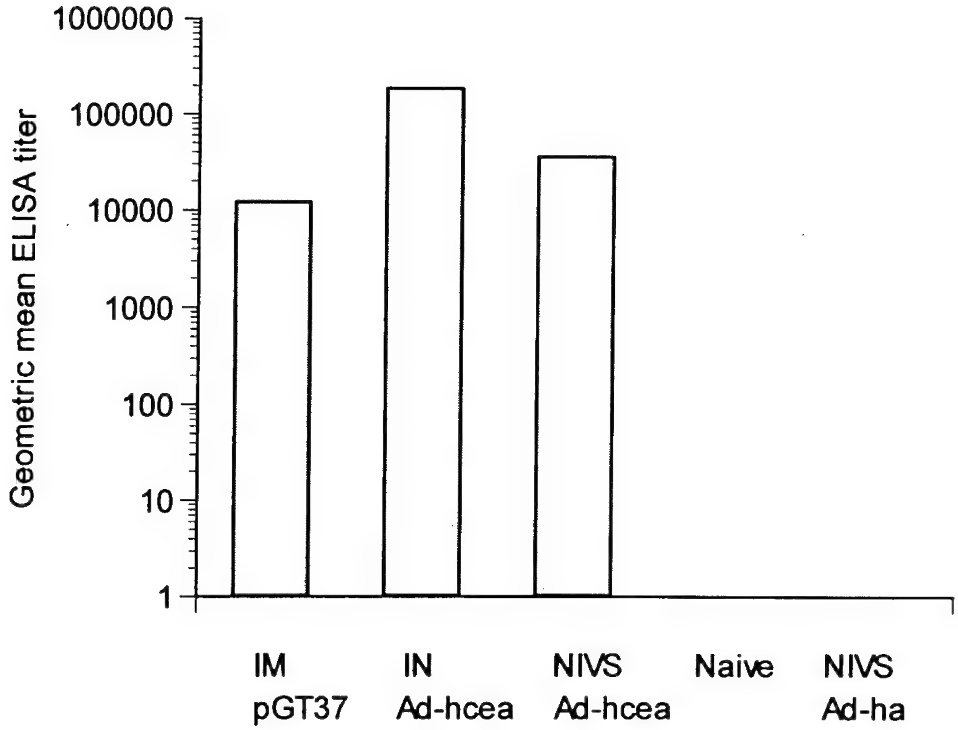

Figure 1. ELISA antibodies generated by the AdCMV-hcea vector in mice. BALB/c mice (3 months old) were immunized by intramuscular (IM) injection of 100 μg of pGT37 DNA, intranasal inoculation (IN) with a dose of 2.5 X 10^7 pfu (plaque-forming units) of AdCMV-hcea, or topical application using a patch by incubating 10^8 pfu of AdCMV-hcea with pre-shaved abdominal skin in a noninvasive mode. For patch-based immunization, the vector was spread as a thin film over naked skin with a piece of the Tegaderm patch (3M). Unabsorbed vectors were washed away in an hour. Each animal was immunized by the specified vector and route for 3 times every 3 weeks. Serum samples were assayed for anti-CEA antibodies 1 week after the last boost. Titers of anti-CEA IgG were determined by ELISA as described (Shi et al., 1999) using purified CEA (CalBiochem) as the capture antigen. Serum samples and peroxidase-conjugated goat anti-mouse IgG (Promega) were incubated sequentially on the plates for 1 hour at room temperature with extensive washing between each incubation. The end-point was calculated as the dilution of serum producing the same OD_{490} as a 1/100 dilution of preimmune serum. Sera negative at the lowest dilution tested were assigned endpoint titers of 100. IM/pGT37, mice immunized by intramuscular injection of pGT37 DNA; IN/Ad-hcea, mice immunized by intranasal inoculation of AdCMV-hcea; NIVS/Ad-hcea, mice immunized by topical application of AdCMV-hcea; Naïve, non-immunized mice as a control group; NIVS/Ad-ha, mice immunized by topical application of an irrelevant vector AdCMV-PR8.ha (an adenovirus vector encoding an influenza hemagglutinin) as a control group. The data was plotted as geometric mean endpoint ELISA titers, where

n=9 for IM/pGT37, n=19 for IN/Ad-hcea, n=9 for NIVS/Ad-hcea, n=10 for naïve, and *n*=10 for NIVS/Ad-ha.

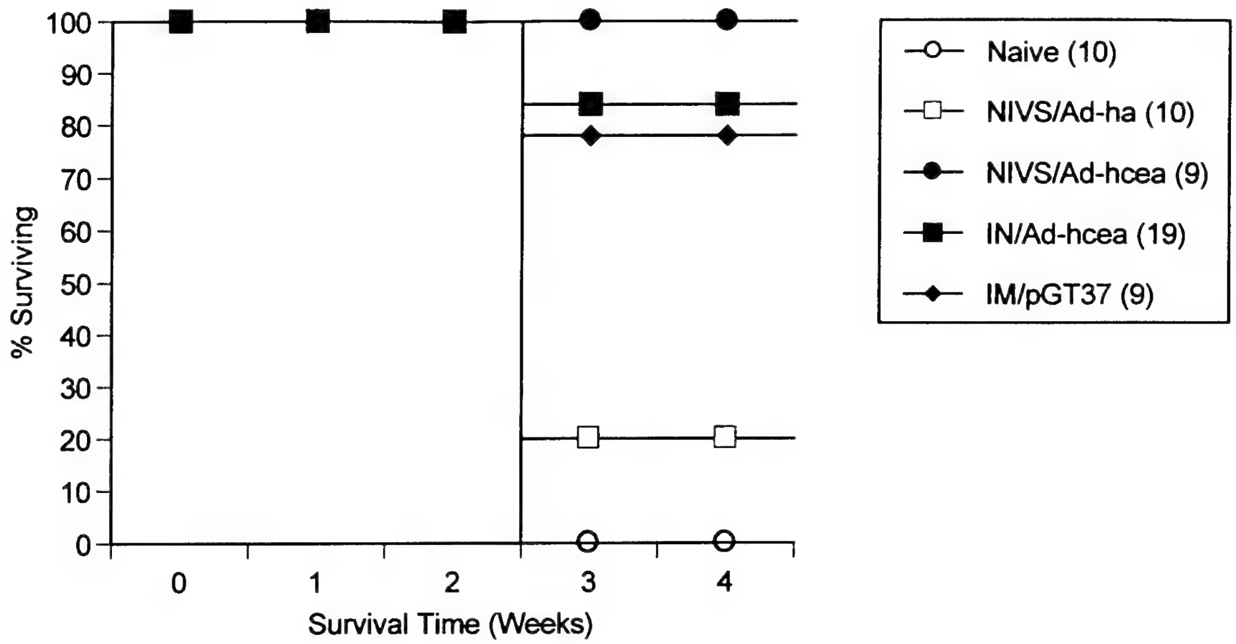

Figure 2. Protection from death after tumor challenge. BALB/c mice (3 months old) were immunized by a variety of vaccination modalities as described in **Figure 1** legend. One week after the last boost, mice were challenged subcutaneously with a lethal dose (3×10^5) of JC-hcea cells and monitored daily for survival. The data was plotted as % survival versus weeks after challenge. Naïve, mice received no vaccines; NIVS/Ad-ha, mice immunized by topical application of AdCMV-PR8.ha; NIVS/Ad-hcea, mice immunized by topical application of AdCMV-hcea; IN/Ad-hcea, mice immunized by intranasal inoculation of AdCMV-hcea; IM/pGT37, mice immunized by intramuscular injection of pGT37 DNA. Numbers in parentheses represented the number of animals for each treatment.

Figure 3. Analysis of antitumor immune responses by in vivo cytotoxicity assay. BALB/c mice (3 months old) were immunized by topical application of AdCMV-hcea as described in **Figure 1** legend. One week after the last boost, 5 X 10^5 JC-hcea cells were implanted onto muscle as a monolayer using a small disk as described (Tang et al., 1996). After 5 days of in vivo growth, the implantation bed was cross sectioned, stained with hematoxylin and eosin, and examined under a light microscope. ***A:*** tissue section from the site of implantation of JC-hcea cells in a naïve mouse 5 days after implantation. Note the presence of a tumor layer on top of muscle (X33). ***B:*** tissue section from the site of implantation of JC-hcea cells in an AdCMV-hcea-based-vaccine-patch immunized mouse 5 days after implantation. Note the eradication of tumor cells and the infiltration of immune effectors into the implantation bed (X33). ***C:*** tissue section as shown in A was visualized at a higher magnification. Note the dominance of JC-hcea cells in the target cell layer with little immune intervention (X132). ***D:*** tissue section as shown in B was visualized at a higher magnification. Note the eradication of tumor cells and evidence for a potent immune intervention (X132).

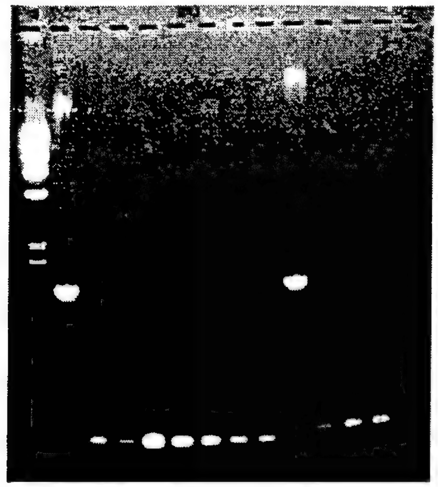

Figure 4. Amplification of foreign DNA in various tissues after localized gene delivery in a noninvasive mode. AdCMV-luc was inoculated onto neck skin in a noninvasive mode as described (Shi et al., 1999). DNA was extracted by DNAZOL (GIBCOBRL), and amplified by the following sets of primers:

Luc5.1: G C G C C A T T C T A T C C T C T A G A
Luc3.1: A C A A T T T G G A C T T T C C G C C C

Luc5.2: G T A C C A G A G T C C T T T G A T C G
Luc3.2: C C C T C G G G T G T A A T C A G A A T

Fb5.1: C C G T C T G A A G A T A C C T T C A A
Fb3.1: A C C A G T C C C A T G A A A A T G A C

Fb5.2: G G C T C C T T T G C A T G T A A C A G
Fb3.2: C C T A C T G T A A T G G C A C C T G T

Luc5.1 and Luc3.1 amplifies the 1.7 Kb full-length luciferase gene; Luc5.2 and Luc3.2 amplifies an 0.52 kb subfragment encompassing the central portion of the luciferase gene; Fb5.1 and Fb3.1 amplifies the 1.7 kb full-length adenovirus type 5 fiber gene; Fb5.2 and Fb3.2 amplifies an 0.55 kb subfragment encompassing the central portion of the fiber gene. Lane M, Molecular weight marker (Lambda DNA cleaved with HindIII); lane 1, full-length luciferase gene amplified by Luc5.1 and Luc3.1 from neck skin DNA 3 hours after NIVS; lane 2, a subfragment of luciferase DNA amplified by Luc5.2 and Luc3.2 from neck skin DNA 3 hours after NIVS; lane 3, a subfragment of luciferase DNA amplified by Luc5.2 and Luc3.2 from neck skin DNA 20 hours after NIVS; lane 4, a

subfragment of luciferase DNA amplified by Luc5.2 and Luc3.2 from mouse ear DNA 20 hours after NIVS; lane 5, a subfragment of luciferase DNA amplified by Luc5.2 and Luc3.2 from abdominal skin DNA 20 hours after NIVS; lane 6, a subfragment of luciferase DNA amplified by Luc5.2 and Luc3.2 from liver DNA 20 hours after NIVS; lane 7, a subfragment of luciferase DNA amplified by Luc5.2 and Luc3.2 from DNA extracted from whole blood 20 hours after NIVS; lane 8, a subfragment of luciferase DNA amplified by Luc5.2 and Luc3.2 from lymph node DNA 7 days after NIVS; lane 9, full-length fiber gene amplified by Fb5.1 and Fb3.1 from neck skin DNA 3 hours after NIVS; lane 10, a subfragment of fiber DNA amplified by Fb5.2 and Fb3.2 from neck skin DNA 3 hours after NIVS; lane 11, a subfragment of fiber DNA amplified by Fb5.2 and Fb3.2 from neck skin DNA 20 hours after NIVS; lane 12, a subfragment of fiber DNA amplified by Fb5.2 and Fb3.2 from mouse ear DNA 20 hours after NIVS. DNA from lymph nodes was extracted by pooling superficial cervical lymph nodes and axillary lymph nodes in DNAZOL solution. DNA was amplified for 35 cycles at optimized annealing temperatures in a Stratagene Robocycler gradient 40 thermal cycler. Amplified DNA fragments were fractionated in 1% agarose gel and stained with ethidium bromide.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
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NAME	POSITION TITLE
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EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

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Baylor College of Medicine, Houston, TX	Postdoc	1988-90	Molecular Biology
Duke University, Durham, NC	Postdoc	1990-91	Gene Therapy
University of Texas-Southwestern Medical Center, Dallas, TX	Postdoc	1991-94	Gene Therapy

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

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1995-99 Assistant Professor, Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, Alabama

1997-Present Vice President and Chief Technical Officer, Vaxin, Inc., Birmingham, Alabama

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Awards and Other Professional Activities:

1994 Young Investigator Travel Grant: $500; American Association for Cancer Research

1994 Start-Up Fund: $300,000; University of Alabama at Birmingham

1997 Scientific Founder; Vaxin, Inc.

1997-99 Investment to Vaxin, Inc.: $733,347; Emerging Technology Partners

1999-00 Investment to Vaxin, Inc.: $1,000,000; Paradigm Venture Partners I, L.L.C.

2000 Allowance of the U.S. patent "Vaccination by topical application of genetic vectors" (U.S. Serial No. 09/402,527)

2000 Year 2000 Wallace H. Coulter Award for Innovation and Entrepreneurship: $100,000; Wallace H. Coulter Foundation

2000-01 Preceptor, Postdoctoral Fellowship: $25,000; Dermatology Foundation

Research Projects Ongoing or Completed During the Last 3 Years:

"Non-invasive delivery of skin-targeted tetanus vaccines"
Principal Investigator: DC Tang, Ph.D.

Agency: National Institutes of Health
Type: Small Business Technology Transfer Program (STTR) Phase I grant (#1-R41-AI-44520-01)
Period: May 1, 1999 to April 30, 2000
Purpose: The major goal of this project is to develop a skin-targeted noninvasive vaccine against tetanus. Because the administration of a "vaccine patch" is simple, economical, painless, and safe, the approach may boost vaccine coverages against tetanus.

"Gene painting as a simple method for vaccinating animals against breast cancer micro-metastases"
Principal Investigator: DC Tang, Ph.D.
Agency: United States Army Medical Research and Materiel Command Breast Cancer Research Program
Type: Idea Award (#DAMD 17-98-1-8173)
Period: October 1, 1998 to September 30, 2001
Purpose: The major goal of this project is to determine whether an antitumor immune response against breast cancer micro-metastases can be elicited by topical application of expression vectors.

"Non-invasive delivery of skin-targeted flu vaccines"
Principal Investigator: DC Tang, Ph.D.
Agency: National Institutes of Health
Type: Small Business Innovation Research Program (SBIR) Phase I grant (#1-R43-AI-43802)
Period: August 1, 1998 to January 31, 1999
Purpose: The major goal of this project is to develop a skin-targeted noninvasive vaccine against influenza. The administration of current influenza vaccine requires needle injection that discourages many people from seeking vaccination-mediated protection. Although nasal spray of an attenuated influenza virus may also be introduced in the foreseeable future, this modality may cause mild flu-like symptoms and is inappropriate for patients with respiratory problems. In contrast to existing influenza vaccines, a skin-targeted and vector-based influenza vaccine patch should not induce any adverse effects because only a limited number of influenza genes will be expressed in the outer layer of skin, which is both a convenient target site for vaccine administration and a very immunocompetent area. An influenza vaccine patch may thus boost vaccine coverages against the virus due to patient comfort.

"Lung cancer immunotherapy by in situ delivery of B7 genes"
Principal Investigator: DC Tang, Ph.D.
Agency: American Lung Association
Type: Research Grant (#RG-167-N)
Period: 1996-1998
Purpose: The major goal of this project is to convert a tumor nodule into a tumor vaccine *in situ* by inoculating adenovirus vectors encoding co-stimulatory molecules into irradiated tumor nodules.

Relevant Publications (Partial Listing)

Tang DC, and Taylor MW. Transcriptional activation of the adenine phosphoribosyltransferase promoter by an upstream butyrate-induced Moloney murine sarcoma virus enhancer-promoter element. J Virol 1990. 64:2907-2911.

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Shi, Z, Curiel DT, and **Tang DC**. DNA-based non-invasive vaccination onto the skin. Vaccine 1999. 17:2136-2141.

Invited Papers:

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Johnston SA, and **Tang DC**. Genetic immunization. In Meyers RA, ed. Molecular Biology and Biotechnology, VCH Publishers, New York 1995, 367-369.

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Vaccine

DNA-based non-invasive vaccination onto the skin

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Abstract

Non-invasive vaccination onto the skin (NIVS) could improve vaccination programs because the procedure requires no specially trained personnel and may eliminate many problems associated with needle injections. There is also evidence that the efficacy of a skin-targeted vaccine may be optimal when the antigen is expressed within the outer layer that is in constant contact with potential pathogens. We report here that non-invasive gene delivery by pipetting adenovirus- or liposome-complexed plasmid DNA onto the outer layer of skin could achieve localized transgene expression within a restricted subset of skin in mice and the elicitation of an immune response against the protein encoded by the DNA. These results provide a proof of principle that NIVS may appear as a novel method for the administration of DNA-based vaccines.

Keywords: DNA-based vaccine; Non-invasive vaccine; Skin-targeted vaccine

1. Introduction

Vaccination usually requires needle injections by medical personnel. Non-invasive vaccination onto the skin (NIVS) by expressing antigens in the outer layer of skin [1] not only may allow the administration of vaccines by individuals without medical training or equipment, but may also elicit more potent immune responses than conventional needle injections given equivalent doses due to the immunocompetence of epidermis along the skin border [2]. We report here that NIVS using DNA-based expression vectors was able to elicit a systemic immune response against the protein encoded by the vector. Unlike inoculation of DNA-based vaccines using a gene gun [3] or a needle [4], the procedure is non-invasive and requires no special skill or equipment. In contrast to NIVS using adenovirus (Ad) recombinants [1], construction and preparation of recombinant plasmid DNA is technically less demanding. Re-vaccination by DNA-based vaccines is also possible [3]. Although NIVS using protein-based vaccines has recently been demonstrated [5], DNA-based vaccines can be purified at lower costs than their protein-based counterparts, and may be able to stimulate a broader spectrum of immune responses for achieving greater efficacy similar to natural infections [6].

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2. Materials and methods

2.1. Cell cultures

Human 293 cells for the propagation of AdCMV-luc [7] were cultured in RPMI medium 1640. W162 cells for the propagation of Ad dl1014 [8] were cultured in DMEM/F12 medium. All media contained 2% fetal bovine serum and 6% calf serum.

2.2. Preparation of DNA/Ad and DNA/liposome complexes

DNA/Ad complexes were prepared by mixing 100 μg plasmid DNA to 1×10^{11} particles of Ad dl1014 for each inoculation. Ad particles were chemically linked to polylysine as described [9] before reacting with DNA. The DNA/Ad complex was further condensed with polylysine. The titer of Ad was determined by absorbance as described [10]. DNA/liposome complexes were prepared by mixing 100 μg plasmid DNA with 100 μg DOTAP/DOPE (1:1; Avanti) for each inoculation. Plasmids were prepared using Qiagen Plasmid Maxi Kits.

2.3. Skin-targeted non-invasive gene delivery

Mice (C57BL/6 strain; 3–8 months old; Jackson) were anesthetized and hair covering a restricted area of neck skin was removed with a WAHL cordless trimmer (Model 8900). The shaved skin was further treated with a depilatory (e.g. Nair) which potentially may facilitate the removal of more cornified epithelium. Ad recombinants, DNA/Ad complexes or DNA/liposome complexes were pipetted into a plastic cylinder (made by drilling a hole through the cap of a Nalgene cryogenic vial) that was glued onto the pre-shaved neck of a mouse. Expression vectors were allowed to incubate with naked skin for 1–18 h. Animal care was in accordance with institutional guidelines.

2.4. Luciferase assay

A piece of excised skin was homogenized and luciferase activity in the skin extract was determined with a luminometer by measurement of integrated light emission for 2 min using the Promega's luciferase assay system.

2.5. Western blot analysis

Sera from tail bleeds were diluted 1:250 and reacted with purified human growth hormone protein (hGH) (CalBiochem) that had been separated in a 12% SDS-polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore) as described [1].

2.6. ELISA for quantitating anti-hGH antibodies

Titers of anti-hGH IgG were determined by ELISA as described [11] using purified hGH as the capture antigen. Serum samples and peroxidase-conjugated goat anti-mouse IgG (Promega) were incubated sequentially on the plates for 1 h at RT with extensive washing between each incubation. The serum samples were diluted in 10-fold increments. The end-point titer was calculated as the dilution of serum producing the same OD_{490} as a 1/100 dilution of preimmune serum.

3. Results

3.1. Skin-targeted non-invasive gene delivery

As an initial step for the development of DNA-based NIVS, we expressed exogenous genes in the skin of mice by pipetting DNA/Ad or DNA/liposome complexes onto naked skin. As shown in Fig. 1, minute amounts of luciferase could be produced in the skin after incubating naked skin with AdCMV-luc particles (an adenovirus vector encoding luciferase driven by the human cytomegalovirus (CMV) promoter) [7], pVR-1216 DNA (a plasmid expression vector encoding luciferase driven by the CMV promoter) complexed with the E4-defective Ad dl1014 [8] or pVR-1216 DNA complexed with DOTAP/DOPE liposomes. No luciferase was detectable in internal organs (e.g. muscle, liver, spleen, heart, lung and kidney) after topical application of expression vectors. The level of transgene expression from Ad recombinants in the skin was on average higher than that from DNA/Ad complexes, which was higher than that from DNA/liposome complexes. The amount of protein produced may potentially be amplified by incubating more vectors with a larger area of skin for a longer period of time. Topical application of pVR-1216 DNA alone without complexing to Ad particles or liposomes produced no measurable luciferase activity in the skin.

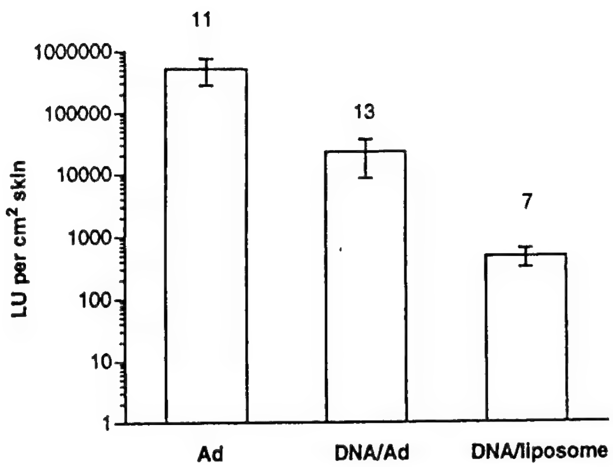

Fig. 1. Skin-targeted non-invasive gene delivery. Mice were inoculated with expression vectors encoding luciferase in a non-invasive mode as described in Section 2. The treated skin was removed at the end of the 18-h incubation period, homogenized and assayed for luciferase activity and background was subtracted from the readings. Mice mock-inoculated or inoculated with DNA alone produced no detectable luciferase activity in the skin. LU, light units; Ad, AdCMV-luc; DNA/Ad, pVR-1216 DNA complexed with Ad dl1014; DNA/liposome, pVR-1216 DNA complexed with DOTAP/DOPE. Results are the mean log[LU per cm^2 skin] ± S.E. (n is shown on top of each column).

3.2. DNA-based NIVS

The expression of transgenes in the skin from topically-applied DNA/Ad or DNA/liposome complexes suggests that these complexes may be formulated as components in skin-targeted non-invasive vaccines. To determine whether the amount of antigen produced in the skin from a topically-applied vector was sufficient for eliciting an immune response, an expression plasmid encoding hGH (pCMV-GH) [3] was complexed with either Ad dl1014 or DOTAP/DOPE liposomes. Mice were subsequently vaccinated by incubating DNA/Ad or DNA/liposome complexes with a restricted subset of pre-shaved skin for 1–18 h. Only a small fraction of vectors could be absorbed by the skin as shown by the ability to retrieve most DNA from the skin surface an hour after incubation. No physical abrasions were found in the skin tissue after incubation, and there was no inflammation associated with the treated skin. Immunized animals were subsequently monitored for the production of antibodies against hGH by assaying sera from tail-bleeds. A month after incubating DNA with naked skin, the test sera from representative mice could react in western blots with purified hGH, but not with bovine serum albumin (BSA) (Fig. 2). Pre-immunization sera, sera from untreated animals and sera from animals vaccinated with irrelevant vectors all failed to react with hGH. Of 7 mice vaccinated by DNA/Ad complexes, all (100%) produced antibodies against hGH within 7 months (Table 1). Of 12 mice vaccinated by DNA/liposome complexes, all (100%) treated animals produced antibodies against hGH (Table 1). The possibility of oral vaccination by ingesting DNA complexes through grooming was eliminated by cleaning the neck skin after removing the DNA-containing cylinder and by mixing naive and vaccinated animals in the same cages. No cross-vaccination between naive and vaccinated mice was ever observed.

The titer of antibodies induced by topical application of DNA/Ad complexes was about 10-fold lower than that elicited by intramuscular injection (IM) of 50 μg of pCMV-GH DNA (Table 1). ELISA showed that DNA/liposome complexes were even less potent than DNA/Ad complexes for eliciting an immune response (Table 1), probably due to the low efficiency of skin-targeted gene delivery (Fig. 1). To demonstrate the feasibility that DNA-based vaccines could re-vaccinate animals in a non-invasive mode, we incubated naked skin of 3 naïve mice with DNA/Ad complexes containing Ad dl1014 complexed with irrelevant plasmid DNA (e.g. pGT37 DNA [12]). As shown in Table 1, antibodies against hGH were still induced when animals with pre-exposure to Ad dl1014 were immunized 9 weeks later by topical application of pCMV-GH DNA/Ad complexes.

4. Discussion

Vaccinating animals or humans by delivering DNA-based vaccines onto the outer layer of skin in a non-invasive mode is an appealing strategy provided that extrinsic antigens can be expressed in viable cells in the authentic skin tissue environment in sufficient quantities for eliciting immunity. We have shown that the production of very small amounts of protein in the skin (Fig. 1) was sufficient for eliciting a systemic immune response (Table 1 and Fig. 2) which may have arisen as a result of antigen expression in a limited number of cells in vivo. The amount of DNA absorbed by the skin during incubation has not been quantitatively measured although it must be small as reflected by the amount of protein produced and the ability to retrieve most DNA from the skin surface. It has been determined that a humoral immune response can be elicited in a mouse by inoculating 40 ng of plasmid DNA into the skin using a gene gun and a cellular immune response can be induced with 0.4 ng of plasmid DNA [13]. Because a fraction of the DNA may be scraped off gold microprojectiles

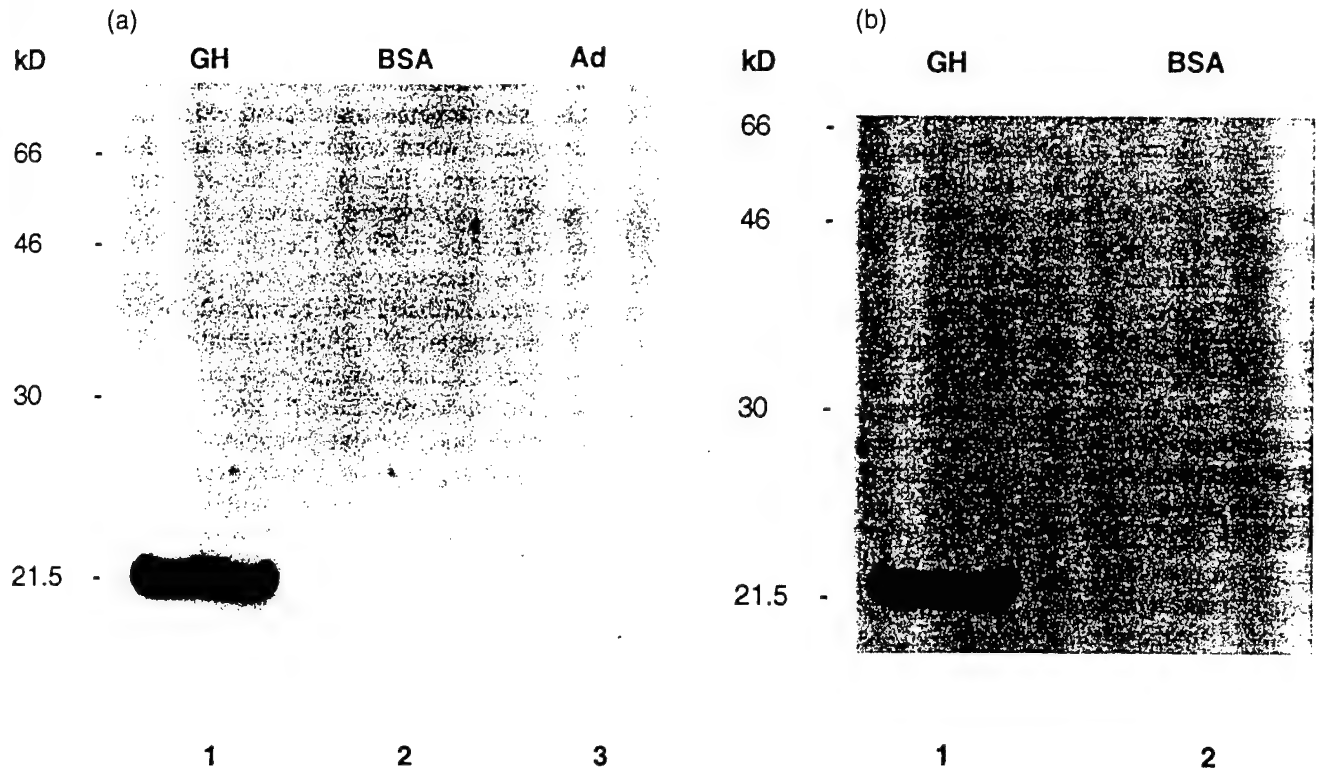

Fig. 2. (a) DNA/Ad-mediated NIVS. Serum from a mouse vaccinated by topical application of pCMV-GH DNA/Ad complexes was analyzed for the presence of anti-hGH antibodies by western blot analysis. Lane 1, hGH (1 μg); lane 2, BSA (1 μg); lane 3, Ad dl1014 (10^{10} particles). (b) DNA/liposome-mediated NIVS. Serum from a mouse vaccinated by topical application of pCMV-GH DNA/liposome complexes was analyzed for the presence of anti-hGH antibodies. Lane 1, hGH (1 μg); lane 2, BSA (1 μg). Animals were immunized by NIVS and western blots were analyzed as described in Section 2.

during a flight when DNA is inoculated into tissues with a gene gun, the minimal amount of DNA that is required for eliciting humoral and cellular immune responses by expressing antigens in the skin may be even less than 40 and 0.4 ng, respectively. The minute amount of DNA that is required for vaccinating an animal via the skin route highlights the immunocompetence of the outer layer of skin and the safety of DNA-based NIVS.

Expression vectors applied onto the skin in a non-invasive mode presumably could penetrate into the body via hair follicles, sweat ducts or minor breaches in the skin. The principal target cells for topically-applied vectors have been identified utilizing either an

Table 1
Summary of the immune responses in mice following DNA-based vaccination

Vector[a]	Pre-exposure to Ad	Mode	Weeks post-immunization	Number of boost	Number of mice producing anti-GH	Anti-hGH IgG serum titer
DNA/Ad	–	NIVS	28	0	2/2	1,000
DNA/Ad	–	NIVS	14	2	5/5	1,000–10 000
DNA/liposome	–	NIVS	28	0	2/2	1,000
DNA/liposome	–	NIVS	22	3	10/10	1,000
DNA	–	IM	12	0	4/4	10,000–100 000
DNA/Ad[b]	+	NIVS	37	1	3/3	10 000

[a] C57 BL/6 mice were immunized by NIVS or IM injection of DNA. DNA/Ad, pCMV-GH DNA complexed with Ad dl1014; DNA/liposome, pCMV-GH DNA complexed with DOTAP/DOPE; DNA, 50 μg pCMV-GH DNA dissolved in saline (1 mg/ml) was injected into the tongue muscle of an anesthetized mouse.[b]Mice were exposed to Ad by topical application of Ad dl1014 complexed with irrelevant DNA (e.g. pGT37 DNA [12]) as described in Section 2. Nine weeks later, animals with pre-exposure to Ad were immunized by non-invasive vaccines containing pCMV-GH DNA complexed with Ad dl1014.

Ad vector encoding β-galactosidase [14] or liposome-complexed plasmid DNA encoding β-galactosidase [15, 16]. Cells within hair follicles [14–16], interfollicular keratinocytes within epidermis [14, 15], as well as dermal fibroblasts [15] appeared as target cells for topically-applied expression vectors. Although the target cells for topically-applied DNA/Ad complexes have not been studied, it is conceivable that they are identical to those transduced by Ad vectors since the tropism of the DNA/Ad complex should be mediated by Ad particles within the complex. Consistent with our finding that no luciferase expression was detected in internal organs including the muscle layer underlying the treated skin, the absence of β-galactosidase-positive muscle cells [14–16] suggests that this non-invasive mode of gene delivery may limit transgene expression within the skin. However, the ability to vaccinate animals by NIVS implies that specific peptide fragments of the exogenous antigens produced in the outer layer of skin may be acquired by professional antigen-presenting cells (APCs) that are able to relocate to lymphoid organs or other sites in the body. Alternatively, a small number of APCs may be directly transfected by topically-applied vectors. Identification and characterization of these putative APCs may provide insights into the mechanisms of NIVS.

The possibility that animals may have been immunized by orally ingesting DNA has been eliminated as described above. It is unlikely that DNA (in the format as described in this report) can immunize animals orally by resisting digestive enzymes found in the gastrointestinal tract. In contrast to the hostile environment that oral vaccines encounter before they battle against pathogens, the skin surface is less destructive to biomolecules. Absorption of DNA by the skin may thus allow epidermal vaccines to be formulated with less sophistication than their oral counterparts. In future vaccination programs, it is conceivable that NIVS and other modes of immunization may complement each other because vaccination via different routes may elicit different immune responses by different mechanisms.

The E1-defective Ad vectors may not be able to vaccinate animals repeatedly as vaccine carriers, attributed to the immunogenicity of Ad vectors which impair Ad-mediated gene expression in animals with pre-existing immunity to Ad [17]. DNA/liposome complexes which do not contain any antigenic components other than the antigen encoded by the DNA should allow continued re-vaccination. It is promising that DNA/Ad complexes containing Ad vectors with reduced immunogenicity (e.g. E4-defective Ad [8], 'gutless' Ad with viral genes deleted [18] or UV-inactivated Ad [10]) will allow re-vaccination or the induction of immune reactivity in animals with pre-exposure to Ad. Given the high skin-targeted transfection efficiency of DNA/Ad complexes when compared to that of DNA/liposome complexes (Fig. 1), a higher antibody titer induced by non-invasive delivery of DNA/Ad complexes over that elicited by their liposome counterparts (Table 1), a persistent wave of in vivo transgene expression from either E4-defective Ad [19] or 'gutless' Ad [20] in immunocompetent animals and the ability to immunize animals with pre-exposure to Ad by DNA/Ad complexes (Table 1), it is likely that DNA/Ad complexes may be able to consolidate the high efficiency of Ad for in vivo gene delivery, the ease with plasmid manipulation and the ability to re-vaccinate animals into one formula for the development of skin-targeted non-invasive vaccines.

Although IM injection of DNA could elicit a more potent immune response than DNA-based NIVS (Table 1), the amount of DNA absorbed by the skin during NIVS was probably only a small fraction of that injected into muscle. NIVS is thus potentially safer than injection of a large dose of DNA into deep tissues. It is conceivable that the efficacy of DNA-based NIVS may be improved by (1) covering a large area of skin for a long period of time, (2) developing a new generation of skin-targeted vectors with a higher in situ transfection efficiency and (3) developing specific adjuvants for NIVS. In contrast to IM injection of pCMV-GH DNA which is capable of eliciting an immune response, intradermal injection of naked DNA appeared as an ineffective vaccination mode for this specific vector [3]. Although direct comparisons between topically-applied DNA/Ad or DNA/liposome complexes and their intradermally-injected counterparts have not been made, it was reported that the deeper the DNA was inoculated into the skin, the less potent the vaccine was [2]. Animals may have evolved an immune surveillance mechanism within epidermis for warding off potential infections along the skin border. We hypothesize that the expression of antigens in a small number of cells within the outer layer of skin can activate the surveillance mechanism and subsequently result in an immune response against the antigen encoded by the vector.

The immunologic competence of the skin, the ease with which genes can be targeted to defined sites on the skin, the rapid turn-over of skin cells, the efficacy of DNA-based vaccines and our finding that animals can be immunized by DNA-based NIVS, may allow for the development of a unique method for vaccination. We envision that skin-targeted non-invasive

vaccines could be delivered by a patch containing a uniform dose of DNA. Since DNA-based NIVS is simple, economical, painless and potentially safe, it may be able to boost vaccine coverages in a wide variety of disease settings.

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